

DESCRIPTION

ANTIBODIES INHIBITING TRANSPORTATION ACTIVITY OF PEPTIDE TRANSPORTER

5 Technical Field

The present invention relates to antibodies that inhibit the transport activity of peptide transporters, and to cell growth inhibitors comprising the antibodies as an active ingredient.

Background Art

10 Mammalian animals need to incorporate nutrients from an external source, and many transport proteins are known to exist in cells. Peptide transporters (peptide transport proteins) transport peptides, and many have been found to date (for example, Non-Patent Documents 1, 2, and 3; and Patent Documents 1, 2, and 3). Peptide transporters can be classified into proteins that import peptides into cells, and proteins that export peptides from cells. They can also be
15 classified by the difference of the energy sources used in transport. Proton-driven peptide transporters, which carry out transport by utilizing the difference in proton concentrations between the inside and outside of a cell, belong to the PTR family (Non-Patent Document 3). Peptide transporters that carry out transport using ATP in the body belong to the ABC family (Non-Patent Document 4).

20 It has been reported that peptide transporters are involved in the transport of not only small-molecule peptides such as dipeptides and tripeptides, but also pharmaceutical agents such as β -lactam antibiotics and ACE inhibitors (Non-Patent Documents 5, 6, 7, 8, 9, and 10).

PepT1 and PepT2 are proton-driven peptide transporters which contribute to the absorption of proteins and the maintenance of peptidic nitrogen sources through uptake of
25 small-molecule peptides into cells. PepT1 and PepT2 are 12-transmembrane proteins, consisting of 708 and 729 amino acids, respectively (Non-Patent Documents 1, 2, and 11).

PepT1 and PepT2 have also been reported to transport pharmaceuticals such as β -lactam antibiotics and bestatin (Non-Patent Documents 12, 13, and 14).

PepT1 is mainly expressed in the small intestine, and its expression in kidney and
30 pancreas has also been confirmed. PepT2 expression has been confirmed in kidney, brain, lung, and spleen. PepT1 and PepT2 have been reported to be localized in the small intestine, as well as in the brush border membrane of renal tubular epithelial cells (Non-Patent Documents 15, 16, 17, and 11).

Furthermore, overexpression of PepT1 in the cell membranes of human pancreatic
35 ductal carcinoma cell lines (Non-Patent Document 18), and PepT2 mRNA expression in human pancreatic ductal carcinoma cell lines (Non-Patent Document 19) have been reported. However,

the involvement of PepT1 and PepT2 in cancer cell growth is not clear, and the question of whether inhibiting the functions of PepT1 and PepT2 will affect cancer cell proliferation has never been discussed.

[Patent Document 1] Japanese Patent Application Kokai Publication No. (JP-A) H6-261761
5 (unexamined, published Japanese patent application)

[Patent Document 2] JP-A H11-172

[Patent Document 3] US 5849525

[Non-Patent Document 1] J. Biol. Chem., 270(12):6456-6463, (1995)

[Non-Patent Document 2] Biochim. Biophys. Acta., 1235:461-466, (1995)

10 [Non-Patent Document 3] Mol. Microbiol., Vol. 16, p825, (1995)

[Non-Patent Document 4] Annu. Rev. Cell. Biol., Vol. 8, p67, (1992)

[Non-Patent Document 5] Ganapathy, Leibach., Curr. Biol. 3, 695-701, (1991)

[Non-Patent Document 6] Nakashima *et al.*, Biochem. Pharm. 33, 3345-3352, (1984)

[Non-Patent Document 7] Friedman, Amidon., Pharm. Res., 6, 1043-1047, (1989)

15 [Non-Patent Document 8] Okano *et al.*, J. Biol. Chem., 261, 14130-14134, (1986)

[Non-Patent Document 9] Muranushi *et al.*, Pharm. Res., 6, 308-312, (1989)

[Non-Patent Document 10] Friedman, Amidon., J. Control. Rel., 13, 141-146, (1990)

[Non-Patent Document 11] Terada, Inui, Tanpakusitsu Kakusan Kousei (Proteins, Nucleic acids, Enzymes), Vol. 46, No. 5, (2001)

20 [Non-Patent Document 12] Saito, H. *et al.*, J. Pharmacol. Exp. Ther., 275, 1631-1637, (1995)

[Non-Patent Document 13] Saito, H. *et al.*, Biochim. Biophys. Acta., 1280, 173-177, (1996)

[Non-Patent Document 14] Terada, T. *et al.*, J. Pharmacol. Exp. Ther., 281, 1415-1421 (1997)

[Non-Patent Document 15] Ogihara, H. *et al.*, Biochem. Biophys. Res. Commun. 220, 848-852, (1996)

25 [Non-Patent Document 16] Takahashi, K. *et al.*, J. Pharmacol. Exp. Ther., 286, 1037-1042 (1998)

[Non-Patent Document 17] Hong, S. *et al.*, Am. J. Physiol. Renal. Physiol., 276, F658-F665 (1999)

[Non-Patent Document 18] Cancer Res., 58, 519-525, (1998)

30 [Non-Patent Document 19] Millennium World Congress of Pharmaceutical Sciences, (2000)

Disclosure of the Invention

The present invention was made in view of such circumstances. Specifically, an objective of the present invention is to provide antibodies that inhibit the transport activity of
35 peptide transporters, and cell growth inhibitors comprising the antibodies as an active ingredient, particularly, cell growth inhibitors for cancers such as pancreatic cancer.

The present inventors discovered that substances which inhibit the transport activity of peptide transporters suppress cell growth. Furthermore, the present inventors discovered antibodies that inhibit the transport activity of peptide transporters. These findings show that cell growth can be suppressed by inhibiting the activity of peptide transporters using the antibodies. Suppression of peptide transporter activity is considered as an important indicator in the development of growth inhibitors against cancer cells and such.

More specifically, the present invention provides:

(1) an antibody that has ability to inhibit the transport activity of a peptide transporter;

(2) the antibody of (1), wherein the peptide transporter is PepT1 or PepT2;

(3) the antibody of (2), wherein the peptide transporter is PepT1;

(4) the antibody of any one of (1) to (3), wherein the antibody is a monoclonal antibody;

(5) a cell growth inhibitor that comprises the antibody of any one of (1) to (4) as an active ingredient;

(6) an anti-cancer agent that comprises the antibody of any one of (1) to (4) as an active ingredient;

(7) the anti-cancer agent of (6), which is pancreatic cancer;

(8) a method for inhibiting the transport activity of a peptide transporter, wherein the method comprises the step of contacting a cell which expresses the peptide transporter with an antibody that binds to the peptide transporter;

(9) the method of (8), wherein the peptide transporter is PepT1 or PepT2;

(10) the method of (9), wherein the peptide transporter is PepT1;

(11) a method for suppressing cell growth, wherein the method comprises the step of inhibiting the transport activity of a peptide transporter by contacting a cell that expresses the peptide transporter with an antibody that binds to the peptide transporter;

(12) the method of (11), wherein the peptide transporter is PepT1 or PepT2;

(13) the method of (12), wherein the peptide transporter is PepT1;

(14) the method of any one of (11) to (13), wherein the cell is a cancer cell; and,

(15) the method of (14), wherein the cancer cell is a pancreatic cancer cell.

The present invention provides antibodies having the ability to inhibit the transport activity of peptide transporters. Peptide transporters of the present invention are not particularly limited; however, they are preferably peptide transporters which incorporate peptides into cells using proton motive force. More preferably, they are PepT1 or PepT2, and most preferably, they are PepT1.

The nucleotide and amino acid sequences of PepT1 and PepT2 are already known (human PepT1: GenBank XM_007063 (J. Biol. Chem., 270(12):6456-6463, (1995)); and human PepT2: GenBank XM_002922 (Biochim. Biophys. Acta., 1235:461-466, (1995))).

The antibodies of the present invention having the ability to inhibit the transport activity of peptide transporters are not particularly limited, as long as they can inhibit peptide transporter-mediated transport (for example, peptide transporter-mediated peptide uptake into cells). Peptide transporter-mediated transport inhibition does not require complete blockage of peptide transport. A decrease in the amount of peptide transported would be sufficient.

There are no limitations on the antibodies of the present invention, as long as they can bind to peptide transporters and inhibit the transport activities of the peptide transporters. Mouse antibodies, rat antibodies, rabbit antibodies, sheep antibodies, camel antibodies, chimeric antibodies, humanized antibodies, human antibodies, and such may be suitably used. The antibodies may be polyclonal or monoclonal, but monoclonal antibodies are preferred in view of the stable production of homogeneous antibodies. Polyclonal antibodies and monoclonal antibodies can be produced by methods well known to those skilled in the art.

Hybridomas that produce monoclonal antibodies can be prepared as follows using basically conventional techniques. Specifically, the hybridomas can be prepared by (1) conducting immunization using a desired antigen, or cells expressing a desired antigen, as the sensitizing antigen according to normal immunization methods; (2) fusing the obtained immunized cells with conventional parent cells by normal cell fusion methods; and (3) screening for monoclonal antibody-producing cells (hybridomas) using normal screening methods. For example, mammalian animals such as mice, rats, rabbits, sheep, and monkeys can be used as animals to be immunized. Antigens can be prepared according to conventional methods such as methods using baculoviruses (*e.g.*, WO 98/46777). When peptide transporters expressed on a

baculovirus membrane are used as an immunogen, gp64 transgenic mice may be used as the immunized animal (International Patent Application No. WO 03/104453).

Hybridomas can be produced, for example, according to the method of Milstein *et al.* (Kohler, G and Milstein, C., Methods Enzymol. (1981) 73: 3-46). When the antigen has low immunogenicity, immunization can be performed by linking it to a macromolecule with immunogenicity, such as albumin.

Recombinant antibodies can also be used, which can be produced by (1) cloning an antibody gene from a hybridoma; (2) incorporating the antibody gene into an appropriate vector; (3) introducing the vector into a host; and (4) producing the recombinant antibodies by genetic engineering techniques (see, for example, Carl, A. K. Borrebaeck, James, W. Larrick, THERAPEUTIC MONOCLONAL ANTIBODIES, Published in the United Kingdom by MACMILLAN PUBLISHERS LTD, 1990). Specifically, cDNAs of the variable regions (V regions) of antibodies are synthesized from hybridoma mRNAs using reverse transcriptase. When DNAs encoding a V region of an antibody of interest are obtained, they are linked to DNAs encoding an antibody constant region (C region) of interest, and then incorporated into expression vectors. Alternatively, DNAs encoding an antibody V region can be incorporated into expression vectors comprising DNAs of an antibody C region. The DNAs are incorporated into expression vectors such that expression is controlled by expression regulatory regions such as enhancers and promoters. Host cells are then transformed with these expression vectors to express the antibodies.

In the present invention, recombinant antibodies artificially modified to reduce xenoantigenicity against humans can be used. Examples of such include chimeric antibodies and humanized antibodies. These modified antibodies can be produced using known methods. A chimeric antibody is an antibody comprising the antibody heavy chain and light chain variable regions of a nonhuman mammal such as a mouse, and the antibody heavy chain and light chain constant regions of a human. A chimeric antibody can be obtained by (1) ligating the DNA encoding a variable region of a mouse antibody to the DNA encoding a constant region of a human antibody; (2) incorporating them into an expression vector; and (3) introducing the vector into a host for production of the antibody.

A humanized antibody, which is also called a reshaped human antibody, is obtained by transplanting a complementarity determining region (CDR) of an antibody of a nonhuman mammal such as a mouse, into the CDR of a human antibody. Conventional genetic recombination techniques for the preparation of such antibodies are known. Specifically, a DNA sequence designed to ligate a CDR of a mouse antibody with the framework regions (FRs) of a human antibody is synthesized by PCR, using several oligonucleotides constructed to comprise overlapping portions at their ends. A humanized antibody can be obtained by (1)

ligating the resulting DNA to a DNA which encodes a human antibody constant region; (2) incorporating this into an expression vector; and (3) transfecting the vector into a host to produce the antibody (see, European Patent Application No. EP 239,400, and International Patent Application No. WO 96/02576). Human antibody FRs that are ligated via the CDR are selected
 5 when the CDR forms a favorable antigen-binding site. As necessary, amino acids in the framework region of an antibody variable region may be substituted such that the CDR of a reshaped human antibody forms an appropriate antigen-binding site (Sato, K. *et al.*, Cancer Res. (1993) 53, 851-856).

Those skilled in the art can determine the CDRs by well-known techniques, for example,
 10 using the database of antibody amino acid sequences prepared by Kabat *et al.* ("Sequences of Proteins of Immunological Interest" U.S. Dept. Health and Human Services, 1983) to analyze the homology.

Methods for obtaining human antibodies are also known. For example, desired human antibodies retaining antigen-binding activity can be obtained by (1) sensitizing human
 15 lymphocytes *in vitro* with antigens of interest or cells expressing antigens of interest; and (2) fusing the sensitized lymphocytes with human myeloma cells such as U266 (see Examined Published Japanese Patent Application No. (JP-B) Hei 1-59878). Alternatively, a desired human antibody can also be obtained by using a desired antigen to immunize a transgenic animal that comprises the entire repertoire of human antibody genes (see International Patent
 20 Application WO 93/12227, WO 92/03918, WO 94/02602, WO 94/25585, WO 96/34096, and WO 96/33735). Furthermore, techniques to obtain human antibodies by panning a human antibody library are known. For example, the variable region of a human antibody is expressed as a single chain antibody (scFv) on the surface of a phage using phage display method, and phages that bind to the antigen can be selected. By analyzing the genes of selected phages, the
 25 DNA sequences encoding the variable regions of human antibodies that bind to the antigen can be determined. If the DNA sequences of scFvs that bind to the antigen are known, appropriate expression vectors to which these sequences are inserted can be constructed to obtain human antibodies. Such methods are well known (see WO 92/01047, WO 92/20791, WO 93/06213, WO 93/11236, WO 93/19172, WO 95/01438, and WO 95/15388).

When the antibody genes have been isolated and introduced into an appropriate host,
 30 hosts and expression vectors can be used in appropriate combination to produce the antibodies. As eukaryotic host cells, animal cells, plant cells, and fungal cells may be used. Known animal cells include (1) mammalian cells such as CHO, COS, myeloma, baby hamster kidney (BHK), HeLa, and Vero cells; (2) amphibian cells such as *Xenopus* oocytes; or (3) insect cells such as sf9,
 35 sf21, and Tn5. Known plant cells include cells derived from the *Nicotiana* genus such as *Nicotiana tabacum*, which can be cultured as callus. Known fungal cells include yeasts such as

the *Saccharomyces* genus, for example *Saccharomyces cerevisiae*, and filamentous fungi such as the *Aspergillus* genus, for example *Aspergillus niger*. Prokaryotic cells can also be used in production systems that utilize bacterial cells. Known bacterial cells include *E. coli* and *Bacillus subtilis*. By transferring the antibody genes of interest into these cells using

5 transformation, and then culturing the transformed cells *in vitro*, the antibodies can be obtained.

Furthermore, the antibody may be an antibody fragment or a modified antibody thereof, as long as it binds to PepT and inhibits its function. For example, the antibody fragment may be Fab, F(ab')₂, Fv, or single chain Fv (scFv) or a diabody, in which Fv from H or L chains are ligated by an appropriate linker. More specifically, the antibody fragment is obtained by (1)

10 treating the antibody with enzymes such as papain and pepsin; (2) transferring it into an expression vector; and then (3) expressing it in an appropriate host cell (see, for example, Co, M. S. *et al.*, J. Immunol. (1994) 152, 2968-2976; Better, M. & Horwitz, A. H. Methods in Enzymology (1989) 178, 476-496, Academic Press, Inc.; Plueckthun, A. & Skerra, A. Methods in Enzymology (1989) 178, 476-496, Academic Press, Inc.; Lamoyi, E., Methods in Enzymology

15 (1989) 121, 663-669; and Bird, R. E. *et al.*, TIBTECH (1991) 9, 132-137). scFv can be obtained by ligating the V regions of the antibody H-chain and L-chain. In scFv, the V regions of the H chain and L chain are ligated via a linker, preferably via a peptide linker (Huston, J. S. *et al.*, Proc. Natl. Acad. Sci. U.S.A (1988) 85, 5879-5883). The V regions of the scFv H chain and L chain may be derived from any of the antibodies described herein. The peptide linker used to

20 ligate the V regions may be, for example, any single-chain peptide consisting of 12 to 19 residues. DNA encoding scFv can be amplified by PCR using as a template either whole DNA, or a partial DNA encoding a desired DNA, selected from a DNA encoding the H chain or the V region of the H chain of the above antibody, and a DNA encoding the L chain or the V region of the L chain of the above antibody; and using a primer pair that defines the both ends. Further

25 amplification can be subsequently conducted using the combination of DNA encoding the peptide linker portion, and the primer pair that defines both ends of the DNA to be ligated to the H chain and the L chain respectively. Once DNAs encoding scFvs are constructed, expression vectors containing the DNAs, and hosts transformed by these expression vectors, can be obtained according to conventional methods. Furthermore, scFvs can be obtained according to

30 conventional methods using the resulting hosts. These antibody fragments can be produced in hosts by obtaining genes encoding the antibody fragments and expressing them in a manner similar to that outlined above. Diabodies are dimers formed by linking two fragments (such as scFvs; hereinafter referred to as diabody-constituting fragments), in which one variable region is linked to the other variable region via a linker or such. Ordinarily, diabodies comprise two VLs and two VHs (Holliger, P. *et al.*, Proc. Natl. Acad. Sci. USA, 90, 6444-6448 (1993); EP 404097;

35 WO 93/11161; Johnson *et al.*, Method in Enzymology, 203, 88-98, (1991); Holliger *et al.*,

Protein Engineering, 9, 299-305, (1996); Perisic *et al.*, Structure, 2, 1217-1226, (1994); John *et al.*, Protein Engineering, 12(7), 597-604, (1999); Holliger *et al.*, Proc. Natl. Acad. Sci. USA., 90, 6444-6448, (1993); Atwell *et al.*, Mol. Immunol. 33, 1301-1312, (1996)).

Antibodies bound to various molecules such as polyethylene glycol (PEG) may be used as modified antibodies. Furthermore, cytotoxic substances such as radioisotopes, chemotherapeutic agents, and bacteria-derived toxins may be attached to the antibodies. Such modified antibodies can be obtained by chemically modifying the obtained antibodies. Methods for antibody modification have been established in the art.

Antibodies used in the present invention may be bispecific antibodies. Bispecific antibodies may be those that comprise antigen binding sites which recognize different epitopes of a peptide transporter molecule. Alternatively, one antigen binding site may recognize a peptide transporter, while another antigen binding site may recognize a cytotoxic substance, such as a radioactive substance, chemotherapeutic agent, or cell-derived toxin. Bispecific antibodies can be produced by joining the HL pairs from two types of antibodies, or by fusing hybridomas that produce different monoclonal antibodies to generate bispecific antibody-producing fusion cells. Furthermore, bispecific antibodies can be produced by genetic engineering techniques.

Alternatively, antibodies with modified sugar chains may also be used in the present invention. Techniques for modifying antibody sugar chains are already known (for example, WO 00/61739, WO 02/31140). The "antibodies" in the present invention include such antibodies.

Antibodies expressed and produced as described above can be purified by conventional methods for purifying normal proteins. Antibodies can be separated and purified by, for example, appropriately selecting and/or combining affinity columns such as a protein A column, or a chromatography column, filtration, ultrafiltration, salt precipitation, dialysis, and such (Antibodies A Laboratory Manual. Ed Harlow, David Lane, Cold Spring Harbor Laboratory, 1988).

Conventional means can be used to measure the antigen-binding activity of the antibodies (Antibodies A Laboratory Manual. Ed Harlow, David Lane, Cold Spring Harbor Laboratory, 1988). For example, an enzyme linked immunosorbent assay (ELISA), an enzyme immunoassay (EIA), a radioimmunoassay (RIA), or a fluoroimmunoassay may be used.

Whether a particular molecule binds to a peptide transporter can be determined using conventional methods. Examples of conventional methods include immunoprecipitation, West-Western blotting, ELISA, EIA, RIA, fluoroimmunoassay, and methods using a biosensor utilizing surface plasmon resonance effect.

Whether antibodies inhibit the transport function of peptide transporters can be determined using known methods, for example, by labeling substrates such as peptides, with a

radioactive substance (e.g., ^{14}C), fluorescent substance, or such, and then measuring the amount of substrate uptake into the peptide transporter-expressing cells (International Patent Application No. WO 03/083116, Zoku Iyakuhin no Kaihatsu 4-Yakubutsu no Seitaimakuyusou to Soshikihyoutekika I, II (Development of Pharmaceuticals 4 - Transport of Pharmaceuticals Through Biological Membranes and Tissue Targeting I, II) (Terada, Hiroshi, Tsuji, Akira. *et al.* ed).

There are no particular limitations on the cells to be targeted by the cell growth inhibitors of the present invention, but cancer cells such as pancreatic cancer cells, liver cancer cells, lung cancer cells, esophageal cancer cells, breast cancer cells, and colon cancer cells are preferred, and pancreatic cancer cells are especially preferred. The cell growth inhibitors of the present invention are used for the purpose of treating and preventing diseases caused by cell growth, and more specifically cancers such as pancreatic cancer.

The antibodies of the present invention may be utilized not only as inhibitors of peptide transporter-mediated transport, but also as cell growth inhibitors, because they are capable of suppressing cell growth as shown in the Reference Examples. The cell growth inhibitors can be administered either orally or parenterally, but are preferably administered parenterally. Specific examples include injections, transnasal administrations, transpulmonary administrations, and transdermal administrations. For example, injections can be administered systemically or locally by intravenous injection, intramuscular injection, intraperitoneal injection, or subcutaneous injection. Furthermore, the method of administration can be selected appropriately according to the age and symptoms of the patient. A single dose can be selected, for example, from within the range of 0.0001 mg to 1,000 mg per kg body weight. Alternatively, the dose can be selected, for example, from within the range of 0.001 to 100,000 mg per patient. However, the dose of a therapeutic agent of the present invention is not limited to these examples. Furthermore, the therapeutic agents of the present invention can be formulated according to standard methods (see, for example, Remington's Pharmaceutical Science, latest edition, Mark Publishing Company, Easton, U.S.A.), and may comprise pharmaceutically acceptable carriers and additives.

Brief Description of the Drawings

Fig. 1 is a graph showing the results of detecting the inhibition of PepT1 activity by anti-human PepT1 monoclonal antibodies in PepT1-expressing viruses. The PepT1 activity on viral membranes was measured as the amount of [^{14}C] glycylsarcosine uptake by the viruses. The data are shown as an average \pm S.D. (n= 3 to 4).

Fig. 2 is a graph showing the PepT1 inhibiting ability of AT-264 in BaF3 cells.

Fig. 3 is a graph showing the cell growth inhibitory effect of AT-264 against human

pancreatic cancer cell line AsPC-1. The data are shown as an average \pm S.D. (n= 3 to 4).

Fig. 4 is a graph showing the PepT2 inhibiting ability of AT-264 in BaF3/PepT2. The data are shown as an average \pm S.D. (n= 3 to 4).

Fig. 5 is a graph showing the cell growth inhibitory effect of AT-264 against human
5 pancreatic cancer cell line BxPC-3. The data are shown as an average \pm S.D. (n= 6).

Fig. 6 shows the nucleotide sequence of the gp64 gene construct.

Fig. 7 is a continuation of Fig. 6.

Fig. 8 shows the structure of the pCAG-gp64 vector construct.

10 Best Mode for Carrying Out the Invention

Hereinafter, the present invention is specifically illustrated with reference to Examples, but it is not to be construed as being limited thereto.

[Example 1] Production of anti-PepT1 antibodies using gp64 transgenic mice (TgM)

15 Primary immunization was performed by subcutaneously injecting a PBS suspension containing PepT1-BV equivalent to a 1 mg protein and 200 ng of pertussis toxin into gp64 TgM (International Patent Application No. WO 03/104453). Subsequent immunizations were performed by subcutaneously injecting PepT1-BV equivalent to a 500 μ g protein (not containing the pertussis toxin) prepared in a similar manner. The final immunization was performed by
20 injecting PepT1-BV equivalent to a 250 μ g protein via the tail vein. Spleen cells were prepared from these mice and were fused with mouse P3U1 cells by a standard polyethylene glycol method. Screening was performed using FACS with BaF/3-PepT1 cells. Furthermore, monoclonal antibodies (clones 113, 119, and 253) which specifically bind to PepT1 were established with a FACS using BaF/3-PepT2 cells.

25

[Example 2] Determining the ability to inhibit PepT1 transport activity

[14 C] Glycylsarcosine was diluted with HBSS (pH6.0) to a final concentration of 50 μ M to prepare a substrate solution. Mouse monoclonal antibodies (clones 119, 253, and 113), which recognize the extracellular region of human PepT1, were diluted with PBS to a final
30 concentration of 200 μ g/mL to prepare antibody solutions. 20 μ L of a solution of budding baculovirus expressing PepT1(50 μ g protein), the N terminus of which is attached to a His-tag, and 20 μ L of each of the antibody solutions were mixed, and pre-incubated at 37°C for one hour. 160 μ L of the substrate solution pre-warmed to 37°C was added to the virus solution to initiate the reaction. One minute later, 1 mL of ice-cold HBSS (pH7.4) (hereinafter referred to as
35 "quenching solution") was added to stop the reaction. The virus-containing reaction solution was immediately filtered by suction through a mixed cellulose membrane filter, and then washed

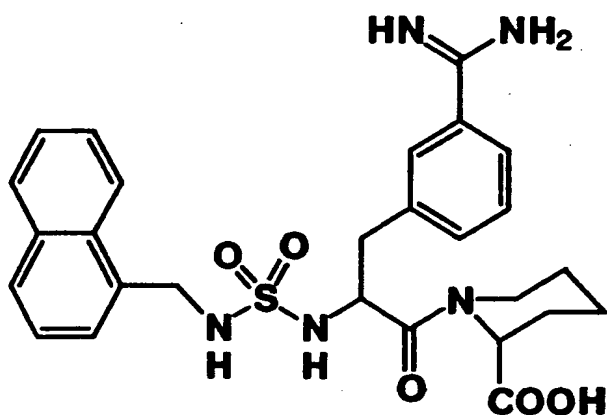
twice with 5 mL of the quenching solution. The membrane filters were transferred into liquid scintillation vials, and 5 mL of Clear-sol I was added to dissolve the filters. After dissolution, the radioactivity on the filters was measured on a liquid scintillation counter. To measure non-specific adhesion to the filter, the quenching solution was added in a similar procedure before the substrate solution was added, and the measured value was subtracted from each of the values obtained from the experiments described above.

The inhibition of PepT1 activity by anti-human PepT1 monoclonal antibodies is shown in Fig. 1. As a control, the PepT1 activity in the absence of the antibodies was indicated as 100. Among the three types of anti-human PepT1 monoclonal antibodies, clone 119 inhibits approximately 20% of the PepT1 activity, and clone 253 inhibits approximately 10% of the activity in comparison to the control. The PepT1 activity inhibition was statistically significant (Student t-test). The above revealed that anti-PepT1 antibodies may have the ability to inhibit PepT1 transport activity.

SEQ ID NO: 1 represents the amino acid sequence of the heavy-chain variable region of clone 119.

[Reference Example 1] AT-264's inhibitory effect on PepT1 activity

AT-264 has a structure represented by the structural formula below. The following experiment confirmed that the compound is a peptide transporter (PepT) inhibitor.



The ability of AT-264 to inhibit PepT1 was examined by forcedly expressing human PepT1 in mouse bone marrow-derived BaF3 cells (hereinafter abbreviated as BaF3/PepT1). The results showed that the uptake of the radioactive substrate [^{14}C] glycylsarcosine into cells was inhibited in a concentration-dependent manner (Fig. 2). Accordingly, AT-264 was found to inhibit PepT1 functions.

[Reference Example 2] The cell growth inhibitory effect of AT-264 against human pancreatic

cancer cell line AsPC-1

AT-264 was dissolved in RPMI1640 - 10 mM Hepes (hereinafter, abbreviated as 'the medium') containing 0.5% ethanol and 0.5% DMSO to prepare 2.5 mM AT-264 solution. Then, this solution was diluted with the medium to prepare 0.625 mM and 0.0625 mM AT-264 solutions.

A 5×10^4 cells/mL solution of human pancreatic cancer cell line AsPC-1 was prepared using a medium containing 50% FBS. This suspension was seeded at 40 μ L/well (2×10^3 cells) onto a 96-well plate pre-coated with Collagen type I, and 160 μ L of the AT-264 solution was added. This was cultured for six days in a CO₂ incubator (on the second day of culture, 100 units/mL penicillin and 0.1 mg/mL streptomycin were added). On the sixth day of culture, the number of viable cells was quantified by MTS assay.

The results of the cell growth experiment are shown in Fig. 3. Cell growth inhibition was confirmed to be approximately 30% in the presence of 2 mM AT-264, and was present even in the presence of 0.5 mM AT-264, although slight. Morphological changes to AsPC-1 were not observed under the microscope, even in the presence of AT-264. Furthermore, the results of RT-PCR indicated that in AsPC-1, PepT1 expression was greater than PepT2. Accordingly, cell growth inhibition by AT-264 was considered to be due to the inhibition of PepT1 function, and not to non-specific cytotoxicity.

[Reference Example 3] Inhibitory effect of AT-264 on PepT2 activity

The ability of AT-264 to inhibit PepT2 was examined using a murine bone marrow-derived cell line BaF3 in which human PepT2 is forcedly expressed (hereinafter, abbreviated as BaF3/PepT2). As a result, the uptake of the radioactive substrate [³H] glycylsarcosine into cells was inhibited in a concentration-dependent manner (Fig. 4).

Accordingly, AT-264 was found to inhibit the function of not only PepT1, but also of PepT2.

[Reference Example 4] Cell growth inhibitory effect of AT-264 on human pancreatic cancer cell line BxPC-3

AT-264 was dissolved in RPMI1640 - 10 mM Hepes with 100 units/mL penicillin and 0.1 mg/mL streptomycin (hereinafter, abbreviated as 'the medium') containing 0.5% ethanol and 0.5% DMSO to prepare 2.5 mM AT-264 solution. Furthermore, this solution was diluted with the medium to prepare 0.625 mM and 0.0625 mM AT-264 solutions.

5×10^4 cells/mL solution of BxPC-3 was prepared using the medium containing 50% FBS. This suspension was seeded at 40 μ L/well (2×10^3 cells) onto a 96-well plate pre-coated with Collagen type I, and 160 μ L of AT-264 solution was added. This was cultured for six days in a CO₂ incubator, and on the sixth day of culture, the number of viable cells was quantified by

MTS assay.

The results of the cell growth experiment are shown in Fig. 5. Cell growth inhibition was confirmed to be approximately 75% in the presence of 2 mM AT-264, and approximately 20% even in the presence of 0.5 mM AT-264. Morphological changes to BxPC-3 were not observed under the microscope, even in the presence of AT-264. Furthermore, the RT-PCR results showed that PepT2 expression was greater than PepT1 expression in BxPC-3. Accordingly, cell growth inhibition by AT-264 was considered to be due to the inhibition of PepT2 function, and not due to cytotoxicity. Therefore, it is clear that substances which inhibit the transport activity of PepT1 or PepT2 also serve as cell growth inhibitors.

[Reference Example 5] Production of gp64 transgenic mice

gp64 transgenic mice were produced according to the method described in International Patent Application No. WO 03/104453. Specifically, the mice were produced by the procedures described below.

1) Construction of the gp64 transgenic vector

PCR was performed using the gp64 gene sequence (GenBank Acc. No. 9627742) as a template, under the conditions described below. As a 5' primer, 64F1 which comprises an EcoRI recognition sequence and a KOZAK sequence at the 5' end was used (Fig. 6); and as a 3' primer, 64R1 which comprises an EcoRI recognition sequence at the 5' end was used (Fig. 7).

The PCR reaction solution had the following composition:

5 μ L of 10 x ExTaq buffer; 4 μ L of dNTP that comes with ExTaq; 1 μ L of 64F1 (10 μ mol/L); 1 μ L of 64R1 (10 μ mol/L); 1 μ L of pBac-N-blue (500 pg/ μ L); 0.5 μ L of ExTaq (5 unit/ μ L); and 37.5 μ L of diw. The reaction sequence was as follows:

94°C 5 min \rightarrow
(94°C 15 sec, 57°C 30 sec, and 72°C 30 sec) x 25 cycles \rightarrow
72°C 7 min \rightarrow
4°C (forever)

The amplified band was subcloned into pGEM-T easy, and was used to transform *E. coli* DH5 α . Colony PCR was performed using the T7 and SP6 primers. Then, nucleotide sequences of the clones that have been confirmed to comprise inserts were analyzed on the ABI Prism 377 DNA sequencer, using the BigDye Cycle Sequence Kit and T7 primer or SP6 primer to identify a clone comprising the gene of interest. After the clone was confirmed not to contain any mutations in its nucleotide sequence, a gp64-comprising fragment was excised from this clone using EcoRI. For transformation of *E. coli* DH5 α , this fragment was inserted into pCAGGS1 which also had been cleaved with EcoRI. The produced clone, which was as

designed, was cultured overnight in 250 mL of LB medium at 37°C, and purified using the Endofree MAXI Kit to yield 581.6 µg of plasmid.

2) Gene introduction

The DNA fragment to be injected was prepared as described below. First, the gp64 gene-harboring pCAGGS vector (pCAG-gp64; Fig. 8) was treated with Sall and PstI to excise a gp64 gene-comprising fragment (approximately 3.8 kb). This fragment (approximately 3.8 kb) was recovered using the Gel Extraction Kit (QIAGEN), and then diluted to 3 ng/µL with PBS to prepare the DNA fragment to be injected.

Mouse pronuclear stage eggs, to which the DNA fragment was injected, were collected as described below. Superovulation treatment was carried out by intraperitoneally administering 5 i.u. of PMSG to female Balb/c mice (Clea Japan) initially, and then intraperitoneally administering 5 i.u. of hCG 48 hours later. These female mice were mated with male mice of the same strain. Mating was confirmed by the presence of a plug the following morning, and the oviducts of mice were perfused to collect the mouse pronuclear stage eggs.

The DNA fragment to be injected was injected into pronuclear stage eggs using a micromanipulator (Modern Techniques in Gene Targeting (Yodosha), 190-207, 2000). The day after the DNA fragment was injected into 373 BALB/c embryos, 216 embryos that had developed to the two-cell stage were transplanted into day 1 pseudopregnant recipient females, approximately ten embryos per oviduct (20 embryos per individual).

[Reference Example 6] Preparation of PepT1-expressing budding baculovirus (PepT1-BV)

The PepT1-expressing budding baculovirus to be used as the immunogen was prepared as described below. PepT1 is a transporter which functions as a membrane protein. The structure of PepT1 is well known (GenBank XM_007063; J. Biol. Chem. 270(12): 6456-6463 (1995)).

A full-length PepT1 gene was isolated from a human renal library using PCR. The transfer vector, pBlueBacHis-PepT1, was prepared by inserting the full-length human PepT1 gene into pBlueBacHis2A (Invitrogen). Using the Bac-N-Blue Transfection Kit (Invitrogen), the transfer vector was then introduced into Sf9 cells, along with a Bac-N-Blue DNA, to produce recombinant viruses for human PepT1 expression. Specifically, 4 µg of pBlueBacHis-PepT1 was added to the Bac-N-Blue DNA, and then 1 mL of Grace's medium (GIBCO) and 20 µL of the Cell FECTIN reagent were added, followed by mixing. The mixture was left to stand at room temperature for 15 minutes, and then added dropwise to 2×10^6 Sf9 cells, which had been pre-washed once with Grace's medium. After letting the cells stand at room temperature for

four hours, an additional 2 mL of complete medium (Grace's medium containing 10% fetal bovine serum (Sigma), 100 units/mL of penicillin, and 100 µg/mL streptomycin (GIBCO-BRL)) was added, and the cells were then incubated at 27°C. Recombinant viruses for human PepT1 expression were then produced by homologous recombination, and purified twice according to the instructions attached to the kit, to obtain a virus stock of the recombinant virus.

Budding viruses which express human PepT1 were prepared as described below. The recombinant virus thus prepared was used to infect 500 mL of Sf9 cells (2×10^6 /mL) at MOI= 5. After culturing at 27°C for three days, the cultured medium was centrifuged at 800 x g for 15 minutes, and the cells and cell debris were removed. The supernatant collected by centrifugation was further centrifuged at 45,000 x g for 30 minutes. The obtained precipitate were suspended in PBS, and then centrifuged at 800 x g for 15 minutes to remove the cell components. The budding virus fraction was obtained by centrifuging the supernatant at 45,000 x g for another 30 minutes, and then resuspending the precipitate in PBS.

15 Industrial Applicability

The present inventors discovered that antibodies which bind to PepT have the ability to inhibit the transport activity of peptide transporters. These antibodies may be utilized as cell growth inhibitors, for example, for treating or preventing cancer.